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Chitosan coated alginate micro particles for oral delivery of antifilarial drug and combinations for intervention in *Brugia malayi* induced lymphatic filariasis.

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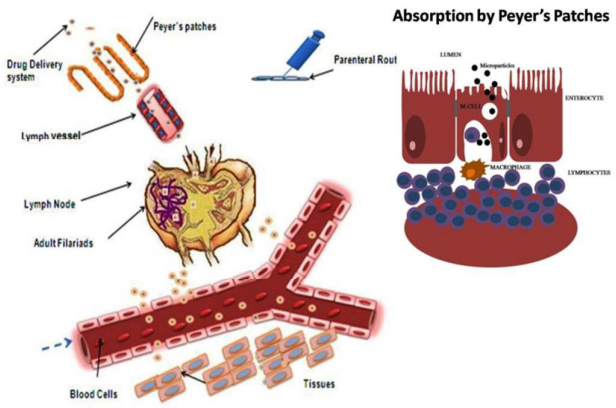
In the present study Chitosan (Chi) coated alginate micro particles (MPs) was prepared by spray drying technique for effective delivery in lymphatics via combination therapy. The optimized MPs were spherical having less than 5µm size with loading and entrapment efficiency of 14.13±1.1% , 41.28±1.5%, for Diethylcarbamazine(DEC), and 14.93 ± 0.58%, 43.51±1.9%, for Doxycycline(DOX) respectively. The physical mixture and drug entrapped MPs showed good stability and no interaction was evident from FT-IR, XRD and thermal analysis. *In vitro* release studies reveal that the drug release pattern was sustained beyond 16h in simulated gastro-intestinal fluids for DEC and DOX from MPs. *In vivo* ileal uptake and histopathological studies in rats divulge uptake of MPs by intestinal epithelium. Further *in vitro* antifilarial activity studies demonstrated notable killing for combination therapy compared to individual treatment with Chi-DOX MPs and Chi-DEC MPs. A combined dose of 25mg/kg and 10 mg/kg in 1:2.5 ratio of DEC and DOX respectively following oral MPs therapy significantly improved the microfilaricidal and macrofilaricidal action *in vitro* estimations. *In vivo* experiments in infected jirds (*Meriones unguiculatus*) were carried out. Macrofilaricidal /Adulticidal action was enhanced by 2 folds Chi-DOX MPs in combination with Chi-DEC MPs, microfilaricidal enhancements of Chi-DEC &Chi-DOX combination is also inferred.

Graphical abstract: Mechanism of targeting of MPs and absorption through peyer's patches, to lymphatics where an adult worm resides.



Introduction

Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease responsible for disabilities in humans[1]. Infection occurs when filarial parasites are transmitted to humans through culex mosquitoes depositing larvae under the skin which migrates to the lymphatic vessels and maturates into adult parasitic worm residing deeply inside the human lymphatic system. Diethylcarbamazine citrate (DEC) is a drug of choice for lymphatic filariasis. DEC, acts by inhibiting mainly the microfilarial stage of the parasite for intervention of the lymphatic filariasis[2]. The second drug Doxycycline (DOX), acts by killing the symbiotic *wolbachia* bacteria residing in the reproductive tract of filarial nematodes such as *Brugia malayi* and *W. bancrofti*. *Wolbachia* plays essential role in worm development, fecundity and survival[3-5] so it is demonstrated that killing the endosymbiont bacteria leads to death of adult worm. The problem with conventional chemotherapy of filariasis is its inaccessibility to the deeply residing worms in lymphatic system[6]. This problem can be effectively tackled by targeted & novel delivery approaches of antifilarial drugs, resulting in elimination of lymphatic filariasis[7]. Microparticulate carriers improve the stability, extend the duration of therapeutic effect and can be orally administered. Oral delivery is physiologically desirable, non-invasive, and having minimal infection risk[8]. Mucoadhesive polymers used in preparation of MPs are reported to maintain contact with intestinal epithelium for prolonged period, and enhance drug passage through and between cells by concentration gradient between delivery system and intestinal membrane. The alginate (Alg) and chitosan (Chi) are ideal

polymer for oral delivery as biocompatible, biodegradable and mucoadhesive polymers. Chitosan is widely used for a microparticulate delivery because it is able to reduce the transepithelial electrical resistance and transiently opening tight conjunction between epithelial cells. Alginate & Chitosan MPs are reported to attribute resistance to gastro-intestinal media, and having potential to be taken up by Peyer's patches[9, 10].It may be directly internalized by enterocytes in contact with intestine, and retention of drugs at their absorptive sites by mucoadhesive polymers. The uptake of MPs by the M cells of the Peyer's patches being absorbed transcellularly, serving as an entry gateway for MPs absorption as pathway through the numerous gut enterocytes. It is reported that positively charged, particles smaller that 10µm, attributes remarkable absorption rate. This absorption pathway opens feasible strategy to overcome antifilarial drug limitations by deeply penetrating inside lymphatic system where adult filariads/worms reside. Peyer's patch, M cells, the lymphoid tissue are adapted to absorb a diversified materials through endocytosis and phagocytosis. Mucosa-associated lymphoid tissue (MALT), interspersed by enterocytes in the follicle-associated epithelium (FAE), can transport versatile drug carriers from the gut lumen to intra-epithelial lymphoid cells by endocytosis and subsequently through the lymphatic system into the blood vessel[11]. DEC & DOX to the gut-associated lymphoid tissue (GALT) by the orally administered MPs may enhance bioavailability of the drugs; impede the enzymatic degradation from enterocytes as well as the first-pass metabolism minimizing the dose and consequently the drug toxicity. Oral delivery of DEC & DOX drugs to the lymphatic system of the filariasis affected patients where the adult filarial worms are residing well by defying the host immune system. It would definitely benefit lymphatic filariasis, by providing improved armament to combat the target parasite. It may prove as the drug carrier to improve the bioavailability of antifilarial drugs directly to adult filarial worms in the human lymphatic system and possibly would lead to a novel strategy for intervention in lymphatic filariasis[12-14].

Materials and Methods

2.1 Materials

Diethylcarbamazine citrate and Doxycycline hyclate were generous gift samples from Fourrts (India) Ltd., Chennai. Sodium alginate (S.D. Fine-Chem. Ltd.), Chitosan (deacetylation, 75-85%; viscosity, 20 to 200 cp), calcium chloride ($CaCl_2$) MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), KH_2P0_4 were purchased from Sigma-Aldrich (USA). Cell culture, RPMI-1640 medium, FITC, dialysis bag (cut off mol. wt. 12 KD) and trypsin from bovine pancreas were procured from Sigma (USA). Well plates for cytotoxicity study were obtained from Greiner Bio (Germany). Water purification system (Milli-Q plus 185, UK) was used for obtaining high quality T.D water. All other reagents and chemicals used were of analytical grade.

Parasites

Sub-periodic strain of *B. malayi* was used in all studies, maintained in jird (*Meriones unguiculatus*) through cyclical transmission by laboratory bred *Aedes aegypti*.

Animals

All experiments were carried out according to Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Protocols were approved by the Institutional Animal Ethics Committee.

Healthy male Wistar rats weighing between 150–200 g were selected for *in vivo* intestinal uptake study; each animal was housed separately and allowed to freely access food and water *ad libitum*.

Jird also called Mongolian gerbils (*Meriones unguiculatus*) ageing 6-8 weeks, maintained at the animal house CSIR-CDRI, Lucknow sub periodic strain of *B. Malayi* maintained through cyclical transmission by laboratory bred *Aedes aegypti*. Jirds were infected by inoculating 100–150 L3 through peritoneal route.

2.2 Preparation of MPs:

MPs were optimized using various concentrations of Alginate, Chitosan, liquid feed rate, spay flow rate, inlet, outlet temperatures. MPs preparation involved spray drying followed by cross linking step. Figure 1 shows the Schematic diagram of the preparation of MPs [15, 16].

2.2.1 Spray drying step:

Drug(DEC/DOX) was dispersed into aqueous solution of sodium alginate (0.5 %, 1 %, 1.5 %)w/v; made by dissolving sodium alginate in triple distilled water under stirring, DEC/DOX homogeneous solution in sodium alginate was spray dried to obtain uncross linked MPs under following operating conditions, having liquid feed rate 5 ml/min, inlet temperature of drying air 125°C, outlet temperature 105°C, spray flow 600 Nlh^{-1}, Aspirator setting-15 was maintained. Free-flowing MPs were collected from the collection chamber. MPs were also collected adhered to the chamber lid, the cyclone chamber taper using a spatula.

2.2.2 Cross linking step:

MPs prepared in first step were suspended in aqueous $CaCl_2$ solution, under mechanical stirring using ultra-turrax at 5000 rpm for 10 min. 1 % (w/v) Chi, solubilised in acetic acid (2%) solutions was mixed to the MPs suspension under mechanical stirring and maintained for 10 min. The cross-linked MPs were then recovered by centrifugation rinsed with T.D water and lyophilized.

2.3 Characterization of MPs

2.3.1 Size and zeta potential

Determination particle size of the MPs using a device (Mastersizer 2000, Malvern Instruments, UK). Briefly, about 5mg of MPs were dry-mixed with an equal amount of sodium lauryl sulphate (SLS) in one ml of T.D. water by vortex-mixing. This slurry was added into the sampling beaker of the instrument till a laser obscuration factor of >10% was achieved. The particle size and size distribution of cross-linked

alginate MPs and cross linked chitosan coated alginate MPs were determined using a laser-based analyzer (Mastersizer2000, Malvern Instruments, U.K). The average size (n=3) in µm were determined for all formulations.

The Zeta Potential is a measure of the electric charge on the surface of the MPs and indicates their physical stability. It measures the electrophoretic mobility of the particles between the electrodes, converted into zeta potential using the Helmholtz–Smoluchowski equation built into the Malvern Zetasizer software. Zeta potential measurements of DEC/DOX loaded and unloaded cross-linked MPs suspended in T.D. water were performed. Zeta potential (n=3) were determined for all formulations[17, 18].

2.3.2Morphology

The optical microscopy (Eclipse E-200, Nikon) was used for initial visualization of MPs. A small drop of MPs suspension was placed on a clean glass slide mounted on the stage of the microscope and observed. Further morphological studies were carried out by scanning electron microscope (SEM) (Leo Electron microscope Ltd, Cambridge, UK). Gold and palladium coated samples were placed over a copper grid and SEM analysis carried out. SEM was used to evaluate the surface texture, shape and degree of clumping of the microspheres. For AFM studies a multi-mode microscope combined with a Gwyddion software version Gwyddion 2.3 (Czech Metrology) was used for data acquisition. The samples were mounted over a cover slip (APER, Italy) and scanned in the tapping mode for topographic images, as well as in the contact mode for force volume analysis. Cantilevers with corresponding properties were used in each model[19].

2.3.3 Thermal analysis, Fourier Transform Infrared (FT-IR) spectroscopy and X-Ray Diffraction Study of Chi-DEC MPs &Chi-DOX MPs

Differential scanning calorimetric analysis of DEC/DOX, blank MPs, Alg-MPs loaded with DEC and DOX respectively were scanned in the range of 50–300°C using a PerkinElmer DSC-4 (USA) at the rate of 10°C/min. The endothermic peaks were measured using PerkinElmer DSC-4 software.

FT-IR study was carried out for DEC, Chi-DEC MPs, DOX, Chi-DOX MPs FT-IR (Shimadzu, Japan). Samples were compressed with KBr on minipress (Jasco, Japan) just before analysis and scanned over 4000 to 4,00 cm^{-1}.The spectra has been presented in Figure 2(a) for DEC and DOX. Further powder X-ray diffraction (PXRD) patterns of DEC, DOX, Alginate polymer, Chi-DEC MPs, and Chi-DOX MPs were recorded on X-ray diffractometer (Rigaku, Japan) equipped with Cu-rotating anode (radiation; λ=1.54 nm) generated at 18 kW. Powder diffractometer operating on Bragg–Brentano geometry was fitted with a curved crystal graphite monochromator in the diffraction beam from the range of 10–60° (2θ)[20].

2.3.4 Drug loading and encapsulation efficiency

DEC/DOX content in MPs was determined by dissolving exactly weighed quantity of samples in 6% (w/v) sodium citrate aqueous solution. The samples were centrifuged at 10000 rpm for 10min. The supernatant was filtered through 0.45 µm (Millipore Filter) and the filtrate was diluted using methanol. DEC/DOX concentrations were measured using a validated reversed phase high performance liquid chromatography (RP-HPLC) method. DEC HPLC analysis was performed using column Lichrocart RP−C8, UV detector (λmax-210 nm), and the mobile phase was a mixture of acetonitrile: phosphate buffer (1:9) v/v, with flow rate of 1.5 ml/min. The solution were filtered and degassed before use via bath sonicator. Chromatography was performed at 30°C[21]. DOX HPLC analysis was performed using column Merck Lichrocart RP-C18 (15 cm×4.6 mm; 5µm), Isocratic flow with flow rate of 1 ml/min. UV Detector (λmax-350 nm), mobile phase consists of Oxalic acid (0.01 M) : Acetonitrile : Methanol (70:18:12). The entrapment efficiency was calculated using the following formula[22].

$$\text{Entrapment Efficiency (\%)} = \frac{(\text{W initial drug} - \text{W free drug}) * 100}{\text{W initial drug}}$$

The drug loading of MPs is generally defined as the amount of drug bound per mass of polymer (usually moles of drug per mg polymer or mg drug per mg polymer). The technique used for this analysis was HPLC after ultracentrifugation.

$$\text{Loading Efficiency (w/w) \%} = \frac{(\text{W initial drug} - \text{W free drug}) * 100}{\text{W microparticles}}$$

2.4 *In vitro* release studies

In vitro release studies has been carried out using USP dissolution apparatus (Lab India, DISSO 2000) type 2 (paddle method) in dissolution media 900 ml at 37±1°C, in 0.1N HCl for 2 hours (h) with pH 1.2 then it was replaced with simulated intestinal fluid (phosphate buffer) having pH 7.4. The stirring speed was set at 50 rpm, at predetermined time intervals 5ml of the sample was withdrawn and replaced with fresh dissolution medium. The samples were analyzed using validated RP-HPLC method. Cumulative percent of drug released was calculated.

In vitro release studies was carried out using USP dissolution apparatus (Lab India, DISSO 2000) type 2 (paddle method) in dissolution media 900 ml at 37±1°C, in 0.1N HCl for 2 h then it was replaced with simulated intestinal fluid(phosphate buffer) having pH 7.4. The stirring speed was set at 50 rpm, at predetermined time intervals 5ml of the sample was withdrawn and replaced with fresh dissolution medium. The samples were analyzed using validated RP-HPLC method. Cumulative percent drug release was calculated. The dissolution data was analyzed using zero order, first order, Higuchi, Korsmeyer-Peppas, Weibull, and Hixson-Crowell cube root law to determine the order and mechanism of drug release[17].

2.5 Stability studies

The stability of Chi-DEC MPs and Chi-DOX MPs was determined in terms of particle size, zeta potential and entrapment efficiency. The formulations were stored and evaluated at 2-

8°C, (25±2°C, 60±5RH) and (40±2°C, 75±5RH) in temperature and humidity conditions[23].

2.6 *In vivo* intestinal uptake study and histopathological study

Male wistar rats (220 ± 20 gm) were divided into two groups having four rats in each group and kept on overnight fasting prior to dosing with Fluorescein isothiocynate (FITC) loaded MPs (FITC-MPs) for uptake studies in Peyer's patches. The fluorescent dye FITC was loaded in to MPs to prepare FITC-MPs using same method as described above for the preparation of MPs, using FITC instead of drug. All the protocol were performed in dark. One mL of FITC loaded formulation (~15mg) was administered orally. The uptake of FITC-MPs in pyloric tissues of ileum of small intestine isolated after sacrificing rat after 3 h of post dosing. About 4-6 cm long part of G.I tract was collected and washed with saline and fixed in formalin for further sectioning using cryostat. Similarly, small intestine sections were collected after 6 h of post dosing respectively and washed with saline fixed, processed and embedded in paraffin wax and 5µm thick sections were cut using microtome (Leica RM, Nussloch, Germany) and images were taken using fluorescent microscope (Leica, Germany). Histopathological study was performed to visualize the localization of the carrier system in Peyer's patches and enterocytes.

2.7 *In vitro* antifilarial activity of the MPs

Stocks solutions of the formulations were prepared by dissolving the sample in one ml of DMSO. Isolation of adult female worms was carried out by the peritoneal washing of infected jirds after 90–150 days of infection then microfilariae (mf) were pelleted after passing the peritoneal washing fluid through 5.0 µm membrane filter. For *in vitro* testing, 10 mf were suspended in 200 µl medium RPMI 1640 /well in a 96 well culture plate. The actively motile female worms were placed in 48 well culture plates (one female worm/ ml/well). Parasites were incubated at 37°C for 72 h in RPMI 1640 medium containing antibiotics (penicillin 100units/ml, streptomycin sulphate 100µg/ml, and neomycin mixture) in presence of serial two fold dilutions of the formulation test samples. Motility of the parasite was observed microscopically at regular time period. At the end of experiment, worms were transferred to fresh drug free medium and kept at 37° C for 1 h to observe reversal in the motility. Worms were processed individually for MTT dye reduction assay for checking their metabolic viability. Experiments were carried out in duplicate and degree of loss in motility as well as percent inhibition in MTT reduction in treated parasites over the untreated controls was assessed. The formulations causing complete irreversible immobility of adult worm along with 50% inhibition in MTT reduction was considered as macrofilaricidal while those bringing about 100% irreversible inhibition in motility of mf were considered microfilaricidal. Minimum concentration causing 100% inhibition in the motility of adult or mf (MIC), 50% inhibitory concentration (IC_{50}) and 50% cytotoxicity on vero cells (CC_{50}) were assessed. The motility scoring of the adult worms as well as of the mf was done as described earlier (Misra et al., 2011) and scored as 0% motility reduction (4+); 1–49% motility reduction (3+); 50–74% motility reduction (2+); 75–99% motility reduction (1+) and 100% motility reduction (Dead). IC_{50} of the active test sample was primarily assessed by using two fold dilutions of each sample starting from MIC using single worm in 48 well plates and 10 mf in 96 well plates in duplicate. IC_{50} was determined by excel based line graphic template after plotting concentrations, Values of each sample versus percentage motility inhibition on x and y axis.CC_{50} of the active test samples was evaluated by carrying out *in vitro* cytotoxicity assay on Vero cells (monkey kidney cell line). Vero cells (10^4/well/100 µl) in 96 well plate were exposed to seven threefold serial dilutions of test sample starting from 300 µg/ml and kept at 37°C in CO_2 incubator. After 72 h, resazurin dye was added and plate was re-incubated for 3–4 h. Fluorimetric reaction was measured using an excitation wavelength of 536 nm and an emission of 588 nm in a fluorimetric plate reader. The safety of the active sample was determined by assessing CC_{50} (50% cytotoxicity on vero cells), IC_{50} (50% inhibitory concentration) and SI (Selectivity Index) (CC_{50}/IC_{50}) to select formulation and formulations with SI ≥10 were considered safe for *in vivo*[17, 24]. IC_{50} determined after 48 h whereas % MTT inhibition was studied after 24 h.

2.8 *In vivo* activity and Assessment of antifilarial activity

In vivo activities were performed in jird transplanted with the adult *B. malayi* in the peritoneal cavity in 6–8 week old male jirds. They were anaesthetized, quickly shaved and a small incision was made on lateroventral region of abdomen to insert adult worms into the peritoneal cavity. Success of transplantation was confirmed by presence of live mf in a drop of peritoneal fluid aspired on day 4 and then these jirds, were ready for experimentation. Formulations activity was tested from day 5 after post worm transplantation and observation was continued till day 45 when jirds were euthanized along with untreated controls. Worms were recovered by peritoneal washing, counted and examined for motility, death or calcification. Live female worms were kept gently in PBS, with pH 7.2. The peritoneal washing fluid stored separately at autopsy on day 45 and then observed microscopically to quantify the effect of drug on peritoneal mf.

Adult *B. malayi* transplanted jird model was used as a *in vivo* model since it helps in determining antifilarial activity quantitatively on adult parasites based on implantation of known number of worms. Additionally large no. of jirds can be transplanted with worms recovered from a single infected jird thus making the screening cost effective. It is easier to separate & sort parasites from peritoneal cavity where L3 are inoculated subcutaneously[17, 24-26].

2.9 Statistical analysis

The statistical data analysis was performed by Prism using one-way Anova (nonparametric) and Dunnett's multiple comparison tests.

Results and discussion

3.1 Characterizations

3.1.1 Size and Zeta potential of MPs

The sizes of the optimized uncross linked-MPs of DEC and DOX were in the range between 1 and 5 µm (90% population). The zeta potential was negative for Alg-MPs and positive for Chi-MPs. The cross linking procedure has not significantly changed the particle size distribution. Chi-DEC MPs & Chi-DOX MPs were having size less than 5µm. The details of UDEC, CDEC, UDOX, CDOX and Blank MPs and size distribution has been given in Table S1. Optical microscopic images showed size, ranging from 2µm up to 5 µm. Sizes between 2 µm and 3µm showed the highest frequency.

3.1.2 Morphology

Morphology of MPs was verified by optical microscopy which revealed spherical MPs and the size obtained was found in agreement with size analysis. It shows both uncross-linked alginate and cross-linked Chi-MPs. By Spray-drying spherical shape uncross linked sodium alginate MPs are obtained (Figure SI). No morphological difference was found in these two different MPs. Therefore results seem to indicate that drug does not affect the morphological characteristics of cross-linked MPs obtained by spray-drying technique. Shape, size and charge of MPs plays critical role during uptakes in Peyer's Patches.

SEM reveals that there are smaller smooth spheres and dimpled particles in formulations which might be due to the rapid evaporation of solvent of the polymer film present at the external surface of droplets during the early stages of spray drying. The subsequent increase in concentration of the polymer at the surface could impede the diffusion of water to the periphery of the droplet and cause a build up of water vapour pressure inside the particle. At a certain point, the film would burst, resulting in particles that have dimples or holes as visualized in Figure 3(b) [18]. AFM results showed as topographic images of the MPs reconfirmed the size and morphology. AFM allows characterizing the physical shape, size and surface structure of the tested formulations. All MPs were spherical in shape having smooth texture. The smooth surface structure can be assumed. The height profiles, making information about vertical dimension, are displayed in the Figure 3(c). Yet from vertical dimension one cannot get horizontal diameter. Reason for the flattening of soft particles can be imaged in the pressure applied by the tip during scanning. In our observations (2µm height/3µm diameter) the average height measured was about 10% of the lateral size, assuming that flattening arises from the tip pressure[19].

3.1.3 FT-IR Study X-Ray Diffraction and Thermal analysis

The presence of vibration in the region of 3,350, 3,050, and 1,650–1,750 cm−1 for –OH, –C–H, and –C=O groups respectively showed presence of DEC. There was neither origin of any new characteristic peaks nor absence of any original characteristic peaks which revealed no incompatibility between drug and excipients. Figure 2(a) shows the characteristic peaks of pure drug, and formulations. Diffractograms of pure drug DEC, pure drug DOX, alginate Polymer, drug loaded Chi-DEC MPs and Chi-DOXMPs are shown in Figure 2(b). The major peaks for DEC was observed at 21°, 24°, 27°, and 32° at an angle of diffraction (2θ) and remained same in physical mixture. There was no sign of formation of any new peak or absence/shift in original characteristic peak. The XRD data revealed the crystalline nature of the drug and physical compatibility between drug and excipients of MPs[20]. XRD Spectra of polymer (alginate) demonstrated absence of any crystalline structure.

DSC thermograms of blank Alg-MPs (a) DEC Pure drug (b) DOX Pure drug, (c) Chi-DEC MPs (d) Chi-DOX MPs, are shown in Figure 2 (c). Melting peaks were shifted in MPs after loading. The endothermic peak of DEC at 225.0°Cwas shifted after loading in MPs and DOX to 250.5°C after loading in MPs. During thermal analysis we have not observed lowering or disappearance of melting peaks suggested that Polymer (alginate & chitosan) did not interact with both DEC and DOX.

3.1.4 Drug loading and %EE:

Drug Entrapment % of formulations of Chi-DEC MPs and Chi-DOX MPs along with size and size distribution are shown in Table S2. Drug loading was calculated with regards to MPs yield obtained after spray drying because MPs yield obtained was less in comparison to amount initially fed into the spray dryer. Drug loading and %EE of Chi-DEC MPs was found to be 14.13 ± 0.52, 41.28±1.5 percent respectively, while loading and %EE of Chi-DOX MPs was found to be 14.93 ± 0.58, 43.51±1.9 respectively.

3.2. *In Vitro* release studies:

In vitro release studies was carried out using USP dissolution apparatus (Lab India, DISSO 2000) type 2 (paddle method) in dissolution media 900 ml at 37±1°C, in 0.1N HCl for 2 h then it was replaced with simulated intestinal fluid (phosphate buffer) having pH 7.4. DEC & DOX loaded cross-linked chitosan coated Alginate MPs.

After 2 h, in simulated gastric fluid (SGF) the release was almost similar to Chi-DEC MPs & DOX MPs batches; however, at pH 7.4 the release was slightly rapid as compared to the Chi-DEC MPs. This could be due to the high water solubility of DEC as compared to DOX. The release pattern Chi-DEC MPs was found to be similar with Chi-DOX MPs in the conditions described above. After 2 h, in SGF the release of DEC was almost same as DOX that is less than 10%. MPs were then transferred to pH 7.4 buffer simulating intestinal conditions.

The cumulative percentage release of DEC and DOX was found to be 52.75±2.21, 49.10±2.11 from Chi-DEC MPs and Chi-DOX MPs respectively after 8 hours at pH 7.4. However, after 12 hours the release of DEC and DOX was observed to be 62.48±2.67, 58.42±2.85 from Chi-DEC MPs and Chi-DOX MPs respectively at same pH as shown in Figure 3(a). The rapid dissolution of drug was detected during the initial transit of the MPs through the gastric cavity. The results are expressed as cumulative percent released in all simulated gastrointestinal fluid (SGIF). Different kinetic models have been employed,

giving linear relationship. The best linearity has been determined in Higuchi's equation plot (DEC=0.969 and DOX=0.929 for MPs) demonstrating the release of drug from the matrix is purely diffusion based phenomenon which followed fickian type of diffusion in which the rate of drug diffusion is lesser than polymer relaxation time. It has been postulated that delaying the drug release could result in higher localization of the drug into the lymphatic system with subsequent reduction in dose of drug with increased specificity. The initial burst release was due to drug adsorbed on the surface of the particles followed by diffusion controlled phenomenon due to swelling of polymeric matrix[17, 27].The R^2 values of release kinetic models applied have been illustrated in Table S5.

3.3. *In vivo* uptake studies in rat ileal sections.

FITC tagged MPs were administered orally after 3 hours of oral administration, FITC labelled MPs were found to be located in luminal mucosal region as evinced by fluorescence. The fluorescence intensity was increased in the same region after 6 hours indicating time dependent uptake. In order to confirm that region of uptake of ileum actually contains peyer's patches and M-cells, the same section stained with hematoxylin (H) & eosin (E). The section showed that the region of ilea collected contains reasonable numbers of payer's patches. Villi of ilea with brush borders were observed with barely identifiable numbers of M-cells. This indicates that uptake of loaded MPs was observed at ileal mucosa; natural location of M-cells which are closely associated with the peyer's patches. Fluorescent microscopic observations of FITC-loaded MPs showed clear adhesion to rat intestinal epithelium, and MPs deeply seen within the intestinal mucosa. Images showing absorption and in vivo uptake studies demonstrated that fluorescently labelled MPs can be taken up by the epithelium of the Peyer's patches[10, 28].

Histology studies of MPs in various part of GI tract were carried out after oral administration. The control did not show any kind of fluorescence in tissue section of small intestine. Furthermore, in Ileum section fluorescent regions could be observed in the connective axis of the villi representing FITC-MPs inside vascular structures. This reveals enhanced uptake of MPs in small intestine. The 6 h post administration exhibited much more increased uptake as evident by high fluorescence in tissue section shown in figure 4[29].

Same sections were used for histology of peyer's patches. Peyer's patches are organized lymphoid nodules and are aggregations of lymphoid tissue that are usually found in the lowest portion of the small intestine, the ileum; as such, they differentiate the ileum. Peyer's patches appear as oval or round lymphoid follicles (analogous to lymph nodes) located in the lamina propria layer of the mucosa and extending into the submucosa of the ileum. The hypertrophy of Peyer's patches causes susceptibility to transmissible spongiform encephalopathies. Peyer's patches are covered by a special epithelium that contains specialized cells called microfold cells (M cells) which sample antigen directly from the lumen and deliver it to antigen-presenting cells (located in a unique pocket-like structure on their basolateral side). They form a lymphoid ring like structure as shown in Figure 5 [30].

3.4.Stability studies

The stability of the optimized MPs formulation was observed by measuring the size, zeta potential and %EE at different time points and at different storage conditions. MPs even after storage for 6 months at 2–8°C, 25 °C / 60 RH. At this stability conditions 40°C / 75 RH small variations in size have been observed. Other formulations have not shown any significant variation in terms of size, zeta potential, and % EE over a period of 6 months of storage in these different stability conditions refer table S2.

3.5. *In vitro* antifilarial activity

The Chi-DEC MPs and Chi-DOX MPs in combination was effective in killing *B. malayi* adult and mf at 25 µg/ml and 10 µg/ml concentration (MIC) respectively. The MPs of DEC, was showing SI value less than 23.8 activities on adults and SI value less than 28.7 activities on microfilariae, The MPs of DOX showing less than 26.66 activity on adult worms and less than 45.63 activity on mf. The MPs in combination of DOX and DEC showed less than 47.66 activities on adults and less than 55.63 activities on mf.

Chi-DEC MPs showed lethal activity of on adult parasite as well as mf with MIC 25.0µg/ml and low IC_{50} values against adult worm and mf being 4.2 and 3.5 µg/ml, respectively. Chi-DOX MPs showed lethal activity on adult parasite as well as mf, MIC of 10.0µg/ml and lowest IC_{50} values against adult worms and mf (1.62 and 1.19 µg/ml, respectively). The CC_{50} (>100 µg/ml) and SI (>10) values of the formulations to be safe for carrying out *in vivo* screening as given in Table S3.

Combination of Chi-DEC MPs+Chi-DOX MPs showed potential lethal activity on adult parasite as well as mf with low IC_{50} values against adult worm and mf (2.10 and 1.9 µg/ml, respectively). The motility scores of the two parasite stages adult and mf along with inhibition percentage in MTT reduction of adult filarial worms treated with Chi-DEC MPs, Chi-DOX MPs and combination of Chi-DEC MPs and Chi-DOX MPs have been shown in Table S3 and Figure 6. The motility scoring and activity evaluation criterion of the adult worms as well as mf was reported earlier and scored as 0% motility reduction (4+); 1–49% motility reduction (3+); 50–74% motility reduction (2+); 75–99% motility reduction (1+) and 100% motility reduction (Dead)[17].

Result indicates that MPs having combinations of DEC+DOX showed additive or summation affects against from adult worms as well as microfilarae of *B. malayi*. The motility scores of the two parasite stages along with percent inhibition in MTT reduction of adults exposed to test samples have been appended in Table S4.

3.6 *In vivo* antifilarial activity, microfilaricidal and macrofilaricidal in jirds

Five different treatments groups of Jird treated with different formulations of Blank MPs, Chi-DEC MPs, Chi-DOX MPs, (DEC+DOX) pure drug combination and (Chi-DEC+Chi-DOX)

MPs combination in amount equivalent to 25 mg/kg and 10 mg/kg respectively. Rationale for taking combination of DEC and DOX and their formulations *in vivo* efficacy studies was based on previous observations (i.e. *in-vitro* efficacy studies).

Macrofilaricidal activity on jirds of Chi-DEC MPs, Chi-DOX MPs, was 33.8 ± 0.48%, 59.0 ±0.25% respectively. (DEC+DOX) MPs and (DEC+DOX) pure drug demonstrated 68.0 ± 0.25 %, 51.1 ± 0.25 % reduction in adult worm percentage respectively, in comparison of the control. Macrofilaricidal activity on jirds, sustained release of Chi-DEC MPs, Chi-DOX MPs, combination of (DEC+DOX) pure drug and (DEC+DOX) MPs was also found effective administration in adult female *B. malayi* 36.0± 0.86%, 66.0±0.47%, 54.9±0.40% and 75.9±0.40% worm death respectively. Besides retarding the worm endurance, both of these regimens affected the fecundity of worms. Chi-DEC MPs, Chi-DOX MPs ,combination of (DEC+DOX) pure drug and (DEC+DOX) MPs administration in adult Male *B. malayi* transplanted jirds exerted 20.0 ± 0.29, 37.14±0.39, 34.70± 0.43 and 85.70± 1.43 macrofilaricidal activity, respectively without any embryostatic actions shown in Figure 7. Conversely, blank MPs at similar doses failed to give any significant detrimental effects on adult worms; however, low degree of worm mortality could be observed in these groups.

The microfilariae density in the treatment groups was significantly lower comparatively without treatment group on day 30. The treated Mongolian gerbil showed a decrease in microfilariae density. Five groups are Chi-DEC MPs, Chi-DOX MPs, (Chi-DECMPs+Chi-DOX MPs combination in 25 and 10 mg/kg dosing respectively), DEC+DOX plain drug combination in a dosing equivalent to 25 mg/kg of DEC and 10 mg/kg of DOX, blank MPs and control group. No. of microfilariae recovered from Mongolian gerbil day 0 was 17.3±1.5, 18.3±1.5, 20.0±1.15, 18.7±1.3, 20.7±0.89 and on day 15 was 14.0±2.0, 15.3±1.7, 15.33±1.2, 21.0±1.7, 23.6±0.88 respectively shown in Figure 8.

The mf density in the treatment groups was significantly lower comparatively without treatment group on day 30. The treated jirds showed a decrease in mf density. Five groups are Chi-DEC MPs, Chi-DOX MPs, (DEC+DOX) Pure drugs, (DEC+DOX) combination MPs, blank MPs as control group. No. of mf recovered from jirds day 0 was 17.3±1.5, 18.3±1.5, 20.0±1.15, 18.7±1.3, 20.7±0.89 and on day 15 was 14.0±2.0, 15.3±1.7, 15.33±1.2, 21.0±1.7, 23.6±0.88 respectively.

Chi-DEC MPs, Chi-DOX MPs, (DEC+DOX) pure drug, (DEC+DOX) combination MPs, and blank MPs treatment mf recovered from jirds day 30 were 12.7±1.7, 15.0±3.2, 13.33±2.0, 20.67±1.45, 24.3±2.2 and on day 45 12.7±1.2, 13.7± 2.9, 12.0± 1.5, 21.0± 0.58, 24.3±2.9 respectively. mf count reduction from pre-treatment level on (day30) preceded by an initial marginal reduction on day 15,was -27.0±7.03,-22.2±8.05,-34.03±6.28, 10.63± 3.17 and 17.27±6.7.Percentage change in mf/10µl blood on day 45 was -26.7±7.7,-30.5±5.40,-28.5±3.40,-40.4±4.30, 11.3±6.55 and 17.5±12.7 shown in Figure 8.

In case of control (Blank MPs) jirds group progressive and steady rise in microfilaraemia was observed. Lymphatic filariasis employing anti-wolbachia therapy along with delivery approaches has offered a comprehensive remedy and drastically improves the efficacy. An increase in the therapeutic index of the antibiotics after entrapment has been well documented in various diseases, DEC/DOX illustrated high encapsulation (50%), demonstrates that entrapment led to slow and continuous release of drug thereby considerably increasing the availability in the circulation for a longer duration over free drug administration. The most noticeable observation was that the Chi-DOX MPs along with Chi-DEC MPs in combination showed significant ($P<0.01$) mf reduction on day 45, while Chi-DOX MPs alone demonstrated significant mf reduction much later, on 45 day. These findings again substantiate earlier observation that Chi-DOX MPs possesses superior microfilaricidal efficacy than Chi-DEC MPs. A combination of Chi-DEC MPs with Chi-DOX MPs in the present study was found to be superior in microfilaricidal efficacious over single entrapped drug. Similarly, synergistic antifilarial activity of liposomal rifampicin, Ivermectin, DOX, and tetracycline in combination with DEC were also well reported [10, 29-31].DEC is, principally, a strong microfilaricidal drug and it imparted an additive effect on both microfilaricidal and macrofilaricidal efficacy of the antibiotic DOX formulations[31]. Apart from the microfilaricidal efficacy, the animals treated with these antibiotic formulations also showed remarkable adulticidal as an activity over free drug administration[32]. The DOX antibiotic exhibited better macrofilaricidal efficacy than Chi-DEC MPs formulation. The results indicate that the encapsulation of drug in MPs directly reaching to the lymphatic site may have effects in its oral absorption and oral bioactivity. It resulted in remarkable reduction of circulating microfilariae and increased adulticidal activity compared with either DEC or DOX alone[33-35].

Conclusions

The spray drying method was found to be rapid & reproducible in producing MPs loaded with antifilarial drugs DEC and DOX in good yields. It has been postulated that delaying the drug release could result in higher localization of the drug into the lymphatic system with subsequent reduction in dose of drug with increased specificity. Uptake studies & drug absorption pathway of MPs by Peyer's patches were demonstrated and visualized by fluorescence microscope. We have observed *in vitro* synergistic antifilarial effects when combination of formulation (Chi-DEC MPs+ Chi-DOX MPs) was used. MPs combinations show potential activity against the adult filarial worms and microfilariae which can kill adult lymphatic filarial parasite. It made the platform to perform the *in vivo* experiments of in filariasis infected animal model jird (Mongolian gerbils).

In vivo antifilarial activity was more pronounced in Mongolian gerbil models, the predilection seat for adult filarial parasites. DEC possesses potential microfilaricidal efficacy with poor macrofilaricidal efficacy in Mongolian gerbil model. DOX shows effective macrofilaricidal efficacy while the combination of DEC and DOX has superior and significant microfilaricidal and macrofilaricidal (adulticidal) effects against *B. malayi*. The

antifilarial activity was substantially improved compared with either drug administered alone. DOX alone was able to reduce wolbachia but combination causes worm mortality at faster pace quantitatively[34].

Parasitic adult worms when exposed to DEC/DOX could hypothesis a simple summation or synergism between the two drugs, but DEC have been reported to potentiate the observed macrofilaricidal activity as it causes degeneration of late embryonic stages and DOX blocks of early embryogenesis and has cause detrimental and lethal effects on filarial adult worms. DEC in some way potentiate these effects. If DEC allowed accumulation of higher concentrations of DOX within the parasite compared with DOX solely, lethal effects independent of wolbachial loads reduction have been observed. Combinational therapy regimes are potentially effective adulticidal treatment having important implications for control of transmission and infections caused by lymphatic filariasis[36]. Filarial nematodes the causative agent of lymphatic filariasis, harbours the endosymbiont bacteria wolbachia, which has been shown to be essential for survival, fecundity, worm growth & development. Mortality of adults directly substantiates the anti wolbachia activity of the DOX. Antifilarial efficacy of these antibiotics is the secondary outcome with primary being symbiotic bacteria wolbachia's death[37]. It may reduce the effective dose of drug required to inhibit filariasis, improves the targeting potential of the developed system[13, 38, 39].

The significant adulticidal effect of a combination DEC/DOX may contribute in its therapeutic interventions & can be used for mass drug administration. The experimental result demonstrates that MPs are the potential drug carrier for an effective lymphatic targeted delivery system. The present findings suggest that chitosan crosslinked alginate DEC/DOX MPs combination attribute remarkable activity against the lymphatic filarial parasite *B. malayi* [4, 40, 41].

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Conflict of Interest statement

The authors declare they have no competing financial interest.

Notes

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Figure legends:

Figure 1: Schematic diagram showing the preparation of microparticles.

Figure 2: (a) FTIR spectra of Pure DEC,DEC MPs, Chi-DEC MPs & Chi-DOX MPs. (b)XRD Graph of Polymer (alginate),Pure DEC, Pure DOX, Chi-DEC MPs & Chi-DOX MPs.**(c)** DSC Curve for DEC, DOX, Chi-DEC MPs & Chi-DOX MPs.

Figure 3: (a) *In Vitro* Release profile of Chi-DEC MPs & Chi-DOX MPs. (b) SEM images and AFM images of Chi-DEC MPs & Chi-DOX MPs.

Figure 4: (a&d): Uptake studies of FITC (FITC loaded Chitosan coated alginate MPs) in small intestine after oral administration at different time intervals (e&f) control (magnification x100).

Figure 5: Histology sections showing peyer's patches and villi stained with Hematoxylin and Eosin (H&E) visualized at 100 X magnifications.

Figure 6: Percentage Inhibition of Adult worms *in vitro* at different concentrations of drug of Chi-DEC MPs, Chi-DOX MPs and Chi-DEC MPs +Chi-DOX MPs (all treatments equivalent to 25μM DEC and 10μM DOX).

Figure 7: Chi-DOX MPs & Chi-DEC MPs,(Chi-DEC+Chi-DOX) MPs combination (DEC+DOX) Plain drug combination and Blank MPs on percentage of macrofilaricidal(adulticidal) efficacy.

Figure 8: Chi-DOX MPs & Chi-DEC MPs, (Chi-DEC+Chi-DOX) MPs combination (DEC+DOX) Plain drug combination, and Blank MPs on microfilaricidal activity.

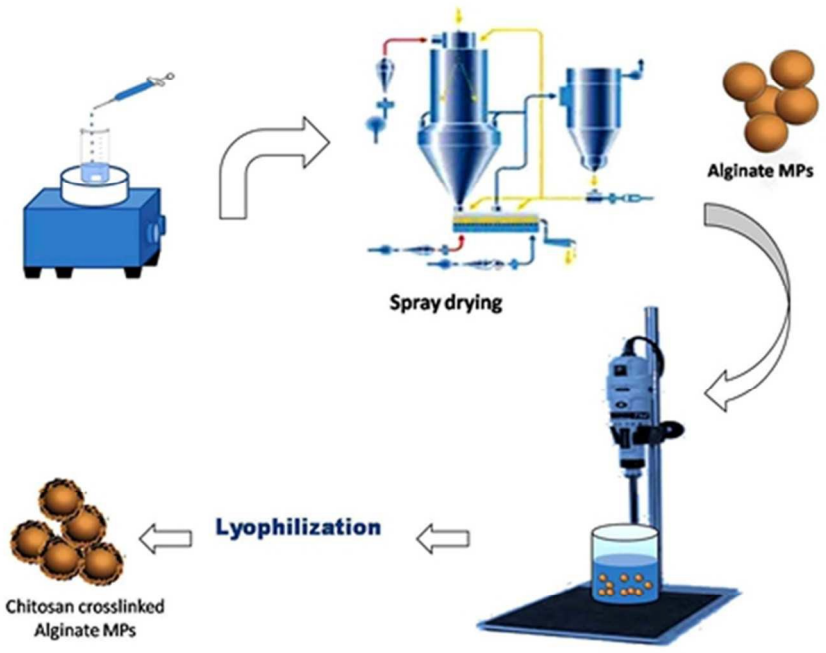

Figure I: Schematic diagram showing the preparation of microparticles.
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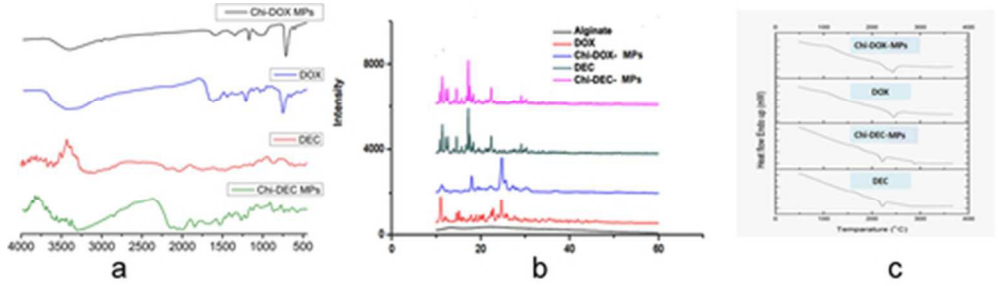

Figure II: (a) FTIR spectra of Pure DEC,DEC MPs, Chi-DEC MPs & Chi-DOX MPs. (b)XRD Graph of Polymer (alginate),Pure DEC, Pure DOX, Chi-DEC MPs & Chi-DOX MPs.(c) DSC Curve for DEC, DOX, Chi-DEC MPs & Chi-DOX MPs.
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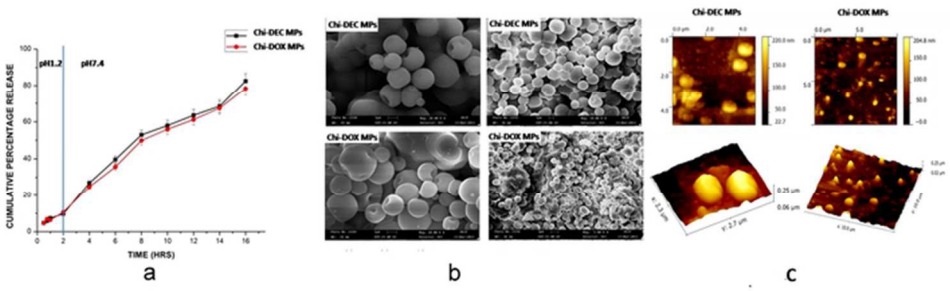


a)in viro dissolution
b) SEm
c) AFM
63x19mm (300 x 300 DPI)

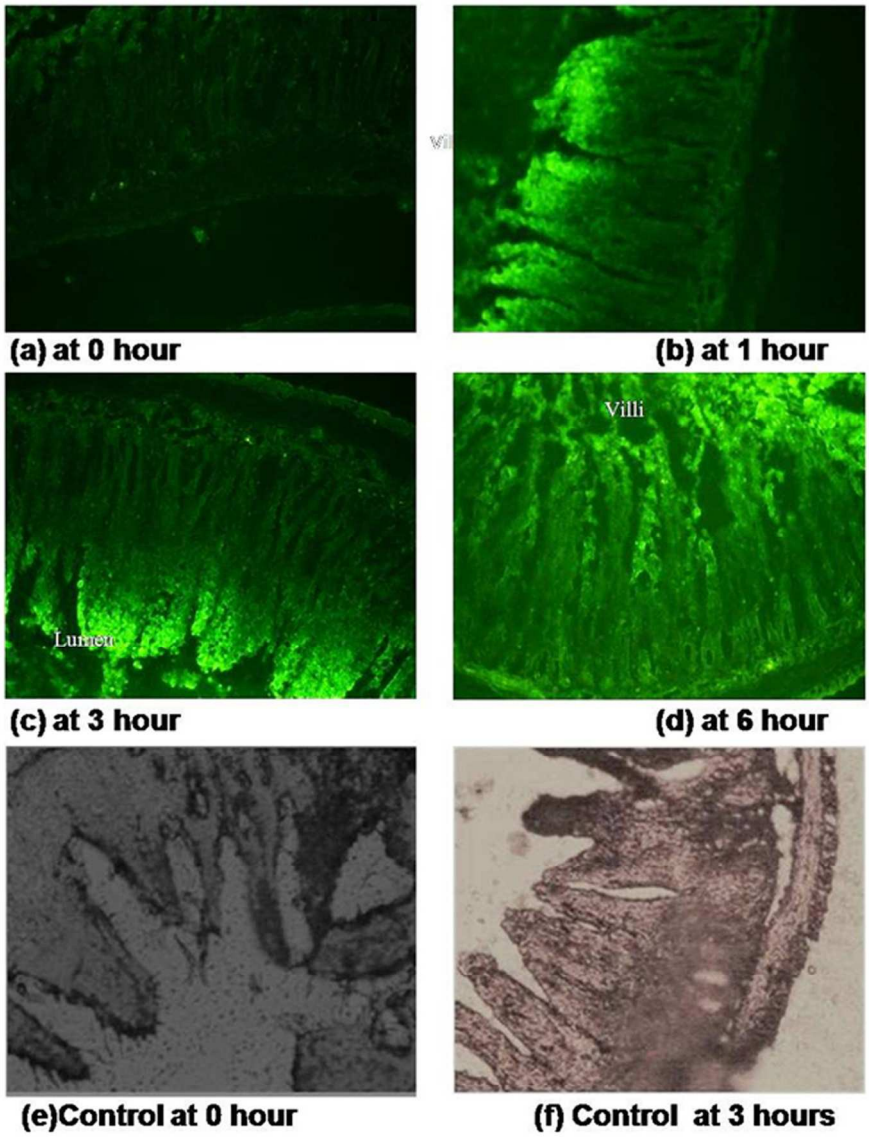
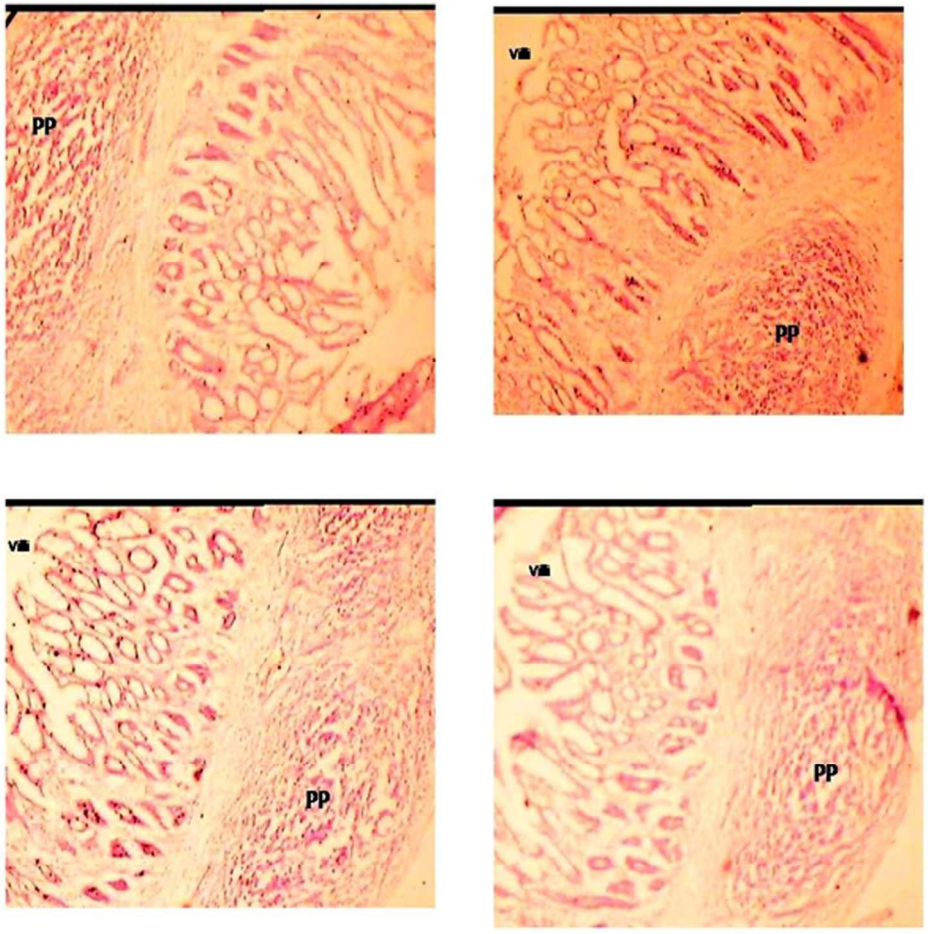
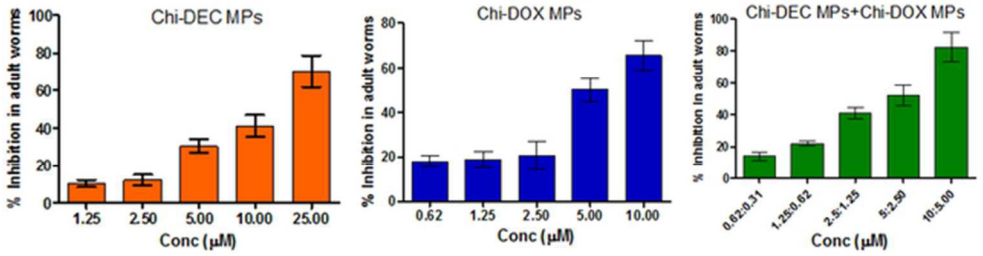


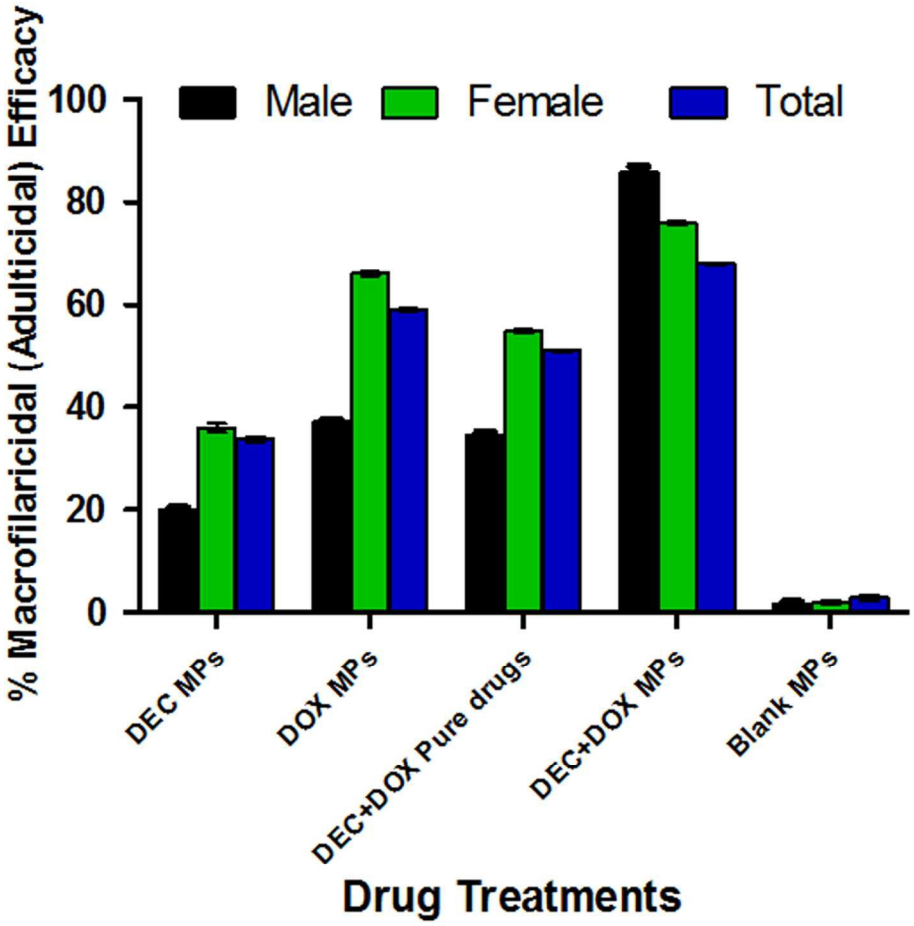
Figure IV: (a&d): Uptake studies of FITC (FITC loaded Chitosan coated alginate MPs) in small intestine after oral administration at different time intervals (e&f) control (magnification x100).
114x152mm (300 x 300 DPI)



Histology sections showing peyer's patches and villi stained with Hematoxylin and Eosin (H&E) visualized at 100 X magnifications.
122x122mm (300 x 300 DPI)



Percentage Inhibition of Adult worms in vitro at different concentrations of drug of Chi-DEC MPs, Chi-DOX MPs and Chi-DEC MPs +Chi-DOX MPs (all treatments equivalent to 25µM DEC and 10µM DOX).
55x14mm (300 x 300 DPI)



Chi-DOX MPs & Chi-DEC MPs,(Chi-DEC+Chi-DOX) MPs combination(DEC+DOX) Plain drug combination and Blank MPs on percentage of macrofilaricidal(adulticidal) efficacy.
110x109mm (300 x 300 DPI)

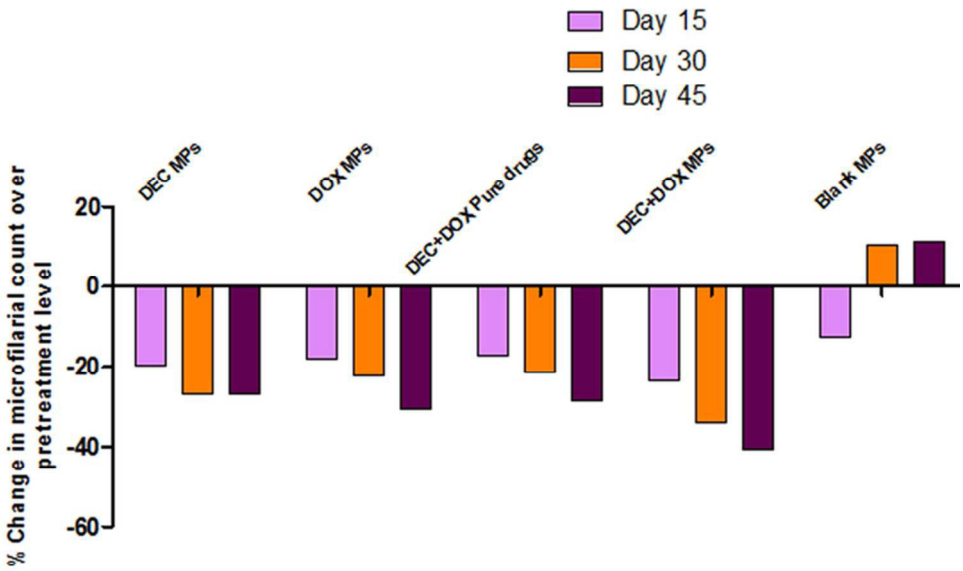


Figure VIII: Chi-DOX MPs & Chi-DEC MPs, (Chi-DEC+Chi-DOX) MPs combination(DEC+DOX) Plain drug combination, and Blank MPs on microfilaricidal activity.
66x44mm (300 x 300 DPI)